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Enhancement of Varicella-Zoster Virus Infection in Cell Lines Expressing ORF4- or ORF62-Encoded Proteins

Sonia Schoonbroodt, Jacques Piette, Laurence Baudoux, Patricia Defechereux, Bernard Rentier, and Marie-Paule Merville

Laboratories of Fundamental Virology and Clinical Chemistry, Department of Microbiology, Institute of Pathology, University of Liège, Belgium

Varicella-Zoster virus (VZV) open reading frames 4 (ORF4) and 62 (ORF62) encode putative immediate-early proteins (ORF4p and ORF62p, respectively) which are strong transactivators of other VZV genes and are involved in the very early stages of viral infection. ORF4p and ORF62p transactivate immediate-early and early gene promoters but have little or no effect on late gene promoters. To investigate the effect of ORF4p or ORF62p overexpression on the viral replication cycle, we constructed Vero cell lines expressing those genes under the control of the human cytomegalovirus major immediate-early promoter. VZV OKA infection of these stably transformed cell lines was followed-up using VZV glycoprotein E (gE) antigen quantification and virus titration. Upon serial passaging of infection in these cell lines expressing functionally active ORF4p or ORF62p, a 5- to 10-fold increase in viral gE antigen production was observed. Viral titers also demonstrated a 2- to 5-fold increase in viral production in these transformed cell lines. These results emphasize the role that both ORF4p and ORF62p play in enhancing the VZV replicative cycle.

KEY WORDS: Varicella-Zoster virus, virus production, immediate-early proteins

INTRODUCTION

Varicella-Zoster virus (VZV) is a member of the human *Alphaherpesvirinae* subfamily and causes chickenpox (varicella) and shingles (zoster). VZV results from a primary infection which is usually contracted during childhood. After a variable latent period, the virus can be reactivated to cause zoster, characterized by inflammation of ganglia and peripheral nerves and associated with acute pain.

In vitro, VZV spreads by direct cell-cell contact and remains highly cell-associated. In cell culture, it is extremely difficult to obtain high titers of cell-free virus, and the various methods used generate a great amount of non-infectious particles. Accordingly, the most extensive analyses have been done for Herpes simplex virus type 1 (HSV-1) genes, and the role of several VZV genes has been deduced from their homology with HSV-1. As for HSV-1, kinetic classes have been assigned to VZV genes, depending upon the order in which they are expressed during the course of infection: immediate-early (IE) genes are expressed first and their products are required for the expression of early (E) and late (L) viral genes. Among the four putative VZV IE proteins, ORF4- and ORF62-encoded proteins (named as ORF4p and ORF62p, respectively) are involved in the very early stages of viral infection.

The VZV ORF4 encodes a putative IE protein, an HSV-1 ICP27 homolog, which seems to act only as an activator of various promoters. ORF4p activates gene expression driven from VZV or other viral promoters [Defechereux et al., 1993; Inschauspe and Ostrove, 1989; Nagpal and Ostrove, 1991], especially the ORF62 promoter, and has little or no effect on the expression of the other three putative VZV IE genes (ORF4, ORF61, ORF63). It also transactivates early gene promoters in a dose-dependent fashion, especially the thymidine kinase gene (ORF36) and the major DNA-binding protein gene (ORF29) promoters, while no activation of late gene promoters was observed [Defechereux et al., 1993].

The ORF62p, identified as the major regulatory protein [Disney and Everett, 1990; Disney et al., 1990; Inschauspe et al., 1989; Perera et al., 1992], has been shown to be the major component of the virion tegument [Kinchington et al., 1992] and is able to transactivate both immediate-early and early gene promoters in transient expression assays [Inschauspe and Ostrove, 1989;

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Address reprint requests to Dr. Marie-Paule Merville, Laboratory of Clinical Chemistry, Institute of Pathology, B23, University of Liège, Sart-Tilman, B-4000 Liège, Belgium.

Inschauspe et al., 1989; Perera et al., 1992]. Possible transactivation of late promoters by ORF62p remains controversial. It was shown that the ORF62p transactivates VZV glycoprotein E (gE) gene promoter [Inschauspe et al., 1989; Perera et al., 1992] and that expression of gE gene was enhanced by synergy between ORF4p and ORF62p [Inschauspe et al., 1989]. It was also demonstrated [Perera et al., 1992] that ORF62p alone could not transactivate other glycoprotein gene promoters or had a weak effect on the gI promoter [Ling et al., 1992]. More recently, no transactivating effect of ORF62p was found on late gene promoters after transient expression assays in Vero cells [Baudoux et al., 1995]. Furthermore, ORF62p has been reported to repress or stimulate its own gene promoter, depending on the experimental conditions [Disney et al., 1990; Perera et al., 1992], and is now considered as a transcriptional activator [Cabirac et al., 1990; Cohen et al., 1993; Everett, 1984; Perera et al., 1992; Perera et al., 1993]. Recent studies [Moriuchi et al., 1994] showed that a high level of ORF62 gene expression markedly enhanced the infectivity of VZV DNA, suggesting that this virion-associated transactivator plays a critical role in initiating the infectious cycle.

To investigate whether ORF4p or ORF62p plays an important regulatory role in amplifying the viral replication cycle, we examined the effects of their overexpression in stably transformed cell lines on the viral production by monitoring the synthesis of a late protein (gE) and measuring viral titers.

METHODS

Cells, Virus Strains, and Infections

Vero and MRC-5 cells, obtained from the American Type Culture Collection (Rockville, MD), were grown in medium 199 and Eagles's minimum essential medium respectively, both supplemented with 10% heat-inactivated fetal calf serum. All cell culture products were purchased from Gibco BRL Laboratories (Paisley, UK). The VZV wild-type strain was isolated by sterile aspiration of vesicular lesions of patients and showed the restriction pattern of VZV strain Ellen. VZV OKA strain was provided by SmithKline Beecham Biologicals (Rixensart, Belgium) as lyophilized cell-free virus. Lyophilized cell-free virus particles, resuspended in 500 μl PGSA buffer (described below), were inoculated to the transfected PBS-washed 35 mm culture for 1 hour and maintained in a medium containing 10% FCS.

Plasmid Construction

ORF4 (the *Nco* I-*Pvu* II fragment from pGi4 [Inschauspe and Ostrove, 1989; Inschauspe et al., 1989]) and ORF62 (the *Avr* II-*Tth* 111 I fragment from p140SV [Disney et al., 1990]) were cloned under the control of the CMV major immediate-early promoter into the *Xba* I site generated in plasmid pRc/CMV (In Vitrogen, San Diego, CA), containing the G418-resistance gene as a selection marker. The resulting plasmids were called pRcORF4 and pRcORF62 respectively. Construction of p62CAT, pTKCAT, and p61CAT has been described elsewhere [Defechereux et al., 1993; Disney et al., 1990; Mc Kee et al., 1990].

Transfections

DNA transfections were carried out using cationic lipid vesicles of Lipofectamine (GibcoBRL, Paisley, UK) according to the manufacturer's instructions.

For transient transfection–infection assays, Vero cells were plated the day before transfection in 35 mm six-well cluster dishes at a density of 2.5×10^5 cells per well. Two μg of pRc/CMV, pRcORF4, or pRcORF62 were mixed with cationic liposomes and layered onto the cells. Twenty-four hours later, the cells were infected with OKA strain. Infected samples were then processed for gE quantification.

For stable transfections, 8×10^5 Vero cells plated in 100 mm Peri dishes were transfected with 10 μg of *Sca* I linearized pRcORF4 or pRcORF62. Twenty-four hours after transfection, cells were trypsinized and 10^6 cells were plated in 100 mm Petri dishes in medium containing G418 (Geneticin; 900 mg/ml [active concentration]; GibcoBRL, Paisley, UK). After approximately 3 weeks, G418-resistant colonies were selected and each of the cloned cell lines examined by immunofluorescence and Western blotting. Transactivating properties of the stably expressed proteins were monitored in transient expression assays where ORF4-expressing cells (named Vero-IE4) were transfected with 2 μg of pTKCAT target plasmid, containing the VZV thymidine kinase (TK) gene promoter and where ORF62-expressing cells (named Vero-IE62) were transfected with 2 μg of p61CAT target plasmid, containing the VZV ORF61 gene promoter. Previous studies in Vero cells have shown that these promoters are transactivated by transiently expressed ORF4p and ORF 62p [Defechereux et al., 1993; Baudoux et al., 1995].

CAT Assays

CAT activity was assayed essentially as described by Neumann et al. [1987]. The cells were washed once in phosphate-buffered saline and resuspended in 0.1 mM Tris-HCl (pH 7.8) in order to be disrupted by three freeze-thawing cycles. Protein concentrations in cell lysates were determined by using the Micro BCA protein assay reagent (Pierce, Rockford, IL). Routinely, 40 μg of proteins in 50 μl of 0.1 mM Tris-HCl (pH 7.8) were mixed to 198 μl of 1.25 mM chloramphenicol and the reaction initiated by addition of 0.5 μCi [^{3}H]-acetyl coenzyme A diluted with carrier acetyl CoA to 0.1 mM (substrate specific activity of 20 mCi/mmol), as suggested by the manufacturer (NEN-Dupont de Nemours, Brussels, Belgium). The reaction was overlaid with 5 ml Econofluor (NEN-Dupont de Nemours, Brussels, Belgium) and the vials were counted continuously in a liquid scintillation counter (miniBETA, LKB, Turku, Finland) to generate kinetics of chloramphenicol acetylation. CAT

activity was quantitated by determination of initial rates of reactions.

Immunolabelling and Western Blotting

1×10^5 Vero-IE4 and Vero-IE62 were grown on sterile glass coverslips, fixed at −20°C in acetone/methanol (v/v) for 20 min and then processed for indirect immunolabelling. The following primary antibodies were used: a polyclonal rabbit antiserum directed against a 13 aa peptide (169 to 181) of the ORF4p and a polyclonal rabbit antiserum directed against the carboxy terminal region of the ORF62p, diluted 1:10 and 1:250 in PBS, respectively. Immune complexes were detected with an FITC-conjugated swine antiserum against rabbit immunoglobulins for ORF4 immunolabelling and a biotinylated swine antiserum against rabbit immunoglobulins, followed by the alkaline phosphatase-coupled avidin-biotin amplification system (ABC-AP) (Dakopatts, Glostrup, Denmark) for ORF62 immunolabeling. In this latter case, New Fuschin (Dakopatts, Glostrup, Denmark) was used as a chromogen in the ABC-AP complex.

To assess the integrity of the proteins expressed in the stably transfected cells, immunoblot analyses were carried out. Cell extracts were prepared in a lysis buffer (Tris-HCl 0.01M; NaCl 0.15 M; deoxycholate de Na 1%; SDS 0.1%; Triton X100 1%; phenylmethyl sulfonyl fluoride 1mM) from a confluent 75 cm^2 culture dish. The lysates were then submitted to a SDS-PAGE electrophoresis before transfer to an Immobilon membrane (Millipore, Bedford, MA). Detection of the VZV ORF4p and ORF62p were performed by using the two antibodies described for the protein immunolocalization experiments. Immunoreactive proteins were detected by the ABC-AP complex described above and by an enhanced chemiluminescence, using commercially available reagents (ECL detection kit; Amersham, Little Chalfont, UK) for the ORF4p and ORF62p immunoblots respectively.

Virus Titration

Cell-free virus was prepared from VZV OKA-infected Vero cells. After aspiration of the medium from a 20 cm^2 tissue culture dish, 1 ml of PGSA buffer (sucrose 0.218 M; KH_2PO_4 0.0038 M; K_2HPO_4 0.0072 M; Na glutamate 0.005 M, BSA 1%, and fetal calf serum 10%) was added, and the infected cell monolayer was collected by scraping with a rubber policeman. The cell suspension was then deposited on 1 ml of glass beads (1 mm in diameter). Cells and glass beads were then subjected to mechanical shaking in a mini-beadbeater (Biospec Products, Bartlesville, OK) at low speed for 10 sec. The glass beads were then separated from the disrupted cell suspension by centrifugation at low speed for 10 min. The supernatant was then submitted to a second centrifugation in the same manner to eliminate cellular debris. The total amount of cell-free virus mechanically released was then titrated on 35 mm MRC-5 monolayer plates. Serial dilutions of the supernatant fluid were inoculated to MRC-5 cells for 1 hr at 37°C and then supplemented with fresh Eagle's minimal essential medium. Plaques were revealed after 48 hr by immunolocalization using an anti-gE monoclonal antibody. The virus titer was expressed as plaque-forming units (PFU) per milliliter of inoculum.

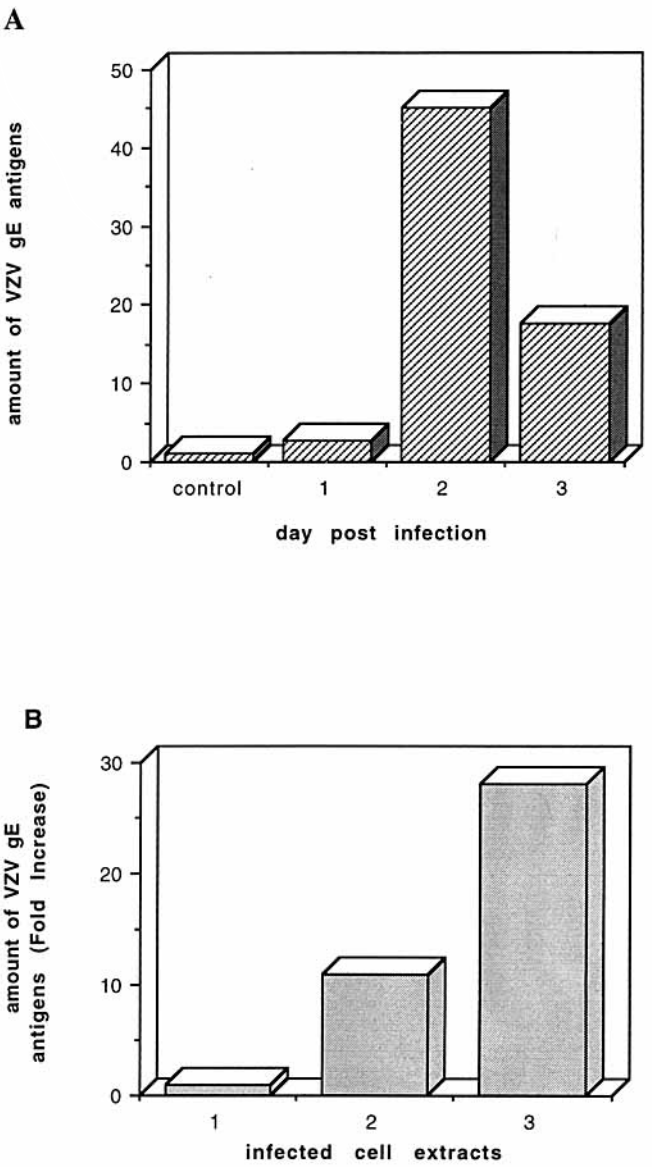
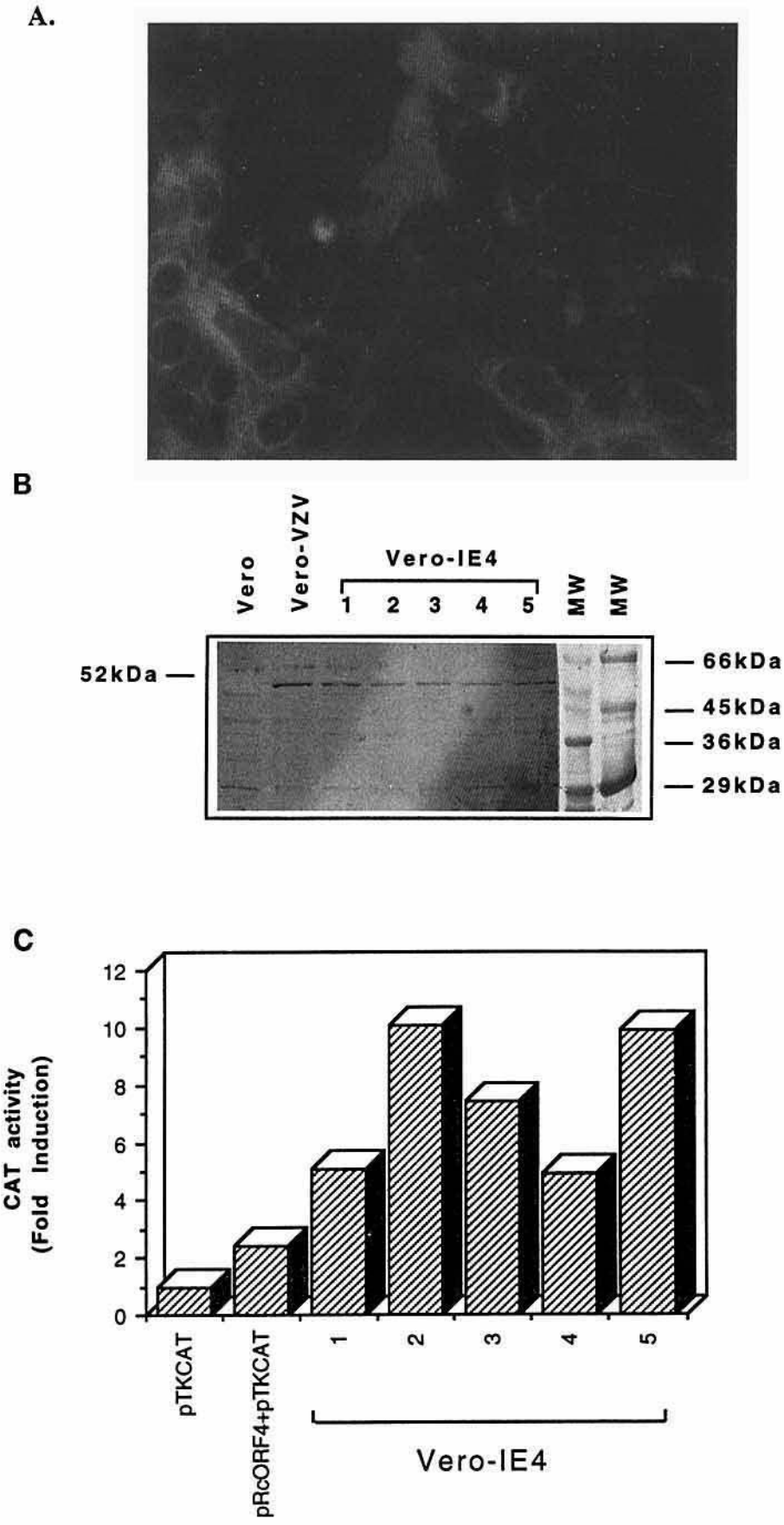


Fig. 1. Vero cell infection by VZV. Amount of gE antigen was measured in infected cell extracts by dot-blotting, using the anti-gE monoclonal antibody VL-8. **A:** Amount of VZV (wild-type strain) gE antigen is followed during the course of infection. Vero cells are infected with an inoculum of VZV infected cells in a ratio 1:4 and harvested 1, 2 and 3 days post infection (p.i.). The "control" lane shows non-infected cells, **lanes 1 to 3** correspond to cells harvested 1, 2, and 3 days p.i. **B:** Infection of transiently transfected Vero cells with VZV OKA strain. Vero cells were either mock-transfected **(1),** transfected with 1 μg of pRcORF4 plasmid **(2),** or with 1 μg of pRcORF62 plasmid **(3).** Twenty-four hr after transfection, the cells were infected with 3×10^3 PFU of VZV OKA strain and harvested 4 days p.i. Data are presented as fold increase in the amount of gE antigen relative to control value, obtained with infected Vero cells.

gE Antigen Quantification

To quantify the increase in gE antigen, VZV OKA-infected cell extracts were dot-blotted on Immobilon membranes, revealed with the anti-gE monoclonal anti-



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body VL-8 [Snoeck et al., 1992] and analyzed by densitometry using a LKB scanner (Pharmacia-Biotech, Uppsala, Sweden). The values described correspond to the average ratio: gE levels in OKA-infected transfected cells/gE levels in OKA-infected control cells.

RESULTS

To determine whether VZV production was enhanced in cell lines expressing ORF4p or ORF62p, experiments were carried out first in transiently transfected Vero cells in order to analyze the effect of overexpressed ORF4p or ORF62p in the course of virus infection. In these transiently transfected cells, 10% of the cells were found positive by immunofluorescence for the detection of ORF4 or ORF62 proteins. Twenty four hr after transfection, each cell culture was infected with 3×10^3 cell-free VZV OKA virus particles. Three to four days after virus inoculation, cytopathic effects (CPE) were observed in ORF4- or ORF62-transfected cells while little or no visible CPE was observed in control infected Vero cells or in Vero cells transfected with 2 μg of pRc/CMV (data not shown). In these preliminary transfection-infection experiments, measurement of virus yields was undertaken by quantification of VZV gE antigen, using the anti-gE monoclonal antibody VL-8. Since it had been observed previously, under the same experimental conditions, that neither ORF4p nor ORF62p have transactivating activities on the VZV gE promoter [Baudoux et al., 1995; Defechereux et al., 1993], we postulate that an increase in the amount of gE antigen reflects an increase in overall viral transcription, suggesting an increase in viral particle production. An 11-fold increase in gE antigen was found in ORF4p-expressing cells and a 28-fold increase in ORF62p-expressing cells (Fig. 1). These results obtained in transient expression assays indicate that expression of ORF4p or ORF62p had a marked effect on the yield of gE antigen.

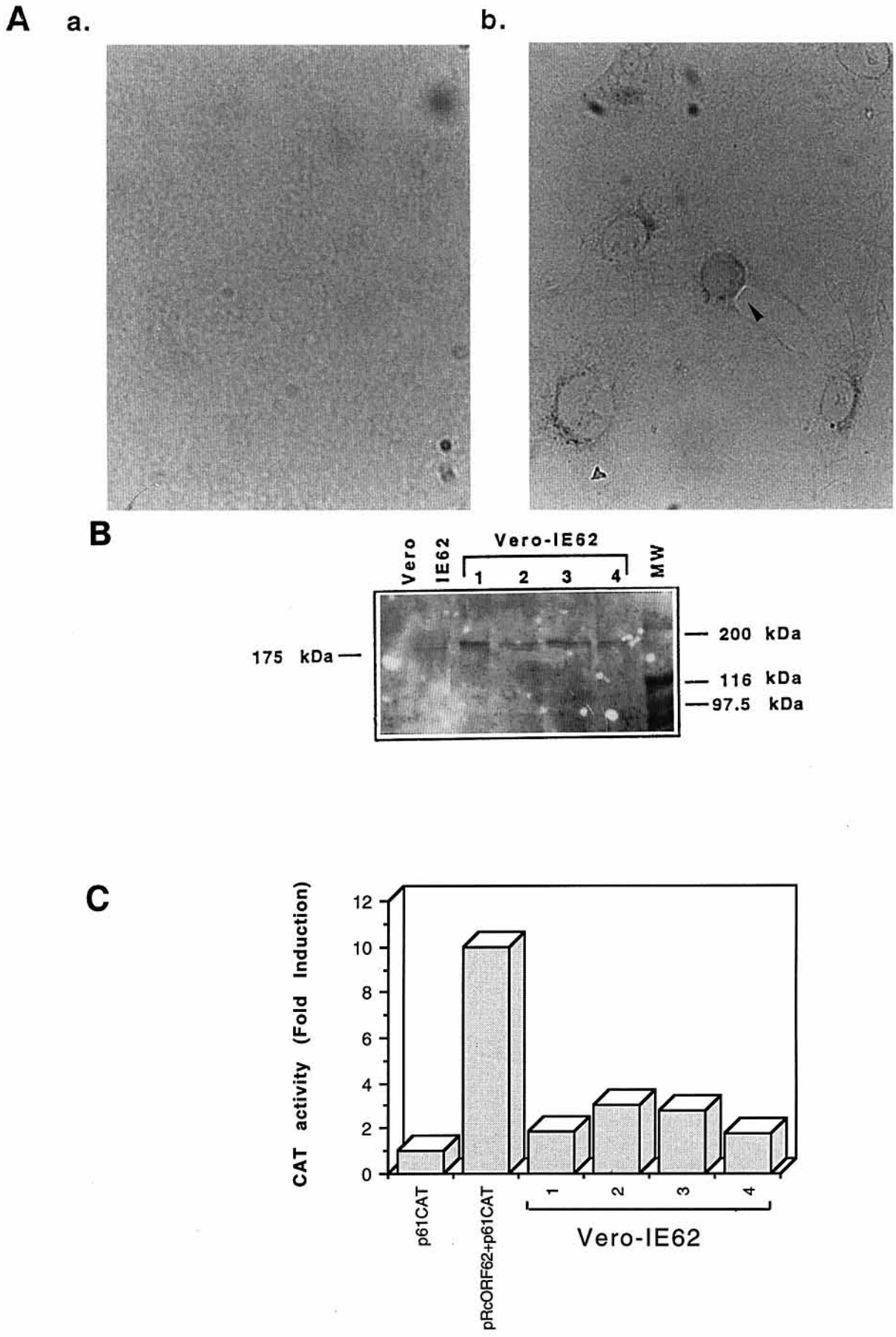
Given the obvious potential interest of stably transformed cell lines, we investigated the establishment and characterization of those cell lines expressing constitutive ORF4p or ORF62p. Vero cells were transfected with pRcORF4 or pRcORF62 (both containing the G418 resistance gene as a selection marker). G418-resistant colonies were examined by immunofluorescence and Western blotting. Transactivating properties of the expressed proteins were monitored in transient expression assays.

Screening for the ORF4p-expressing cell lines allowed us to select one cell line, named 3A14, where 0.5–2.5% of the cells were positive by immunofluorescence for the expression of ORF4p. In these ORF4p-expressing cells (Vero-IE4), the cellular localization of the protein was similar to that observed in control VZV-infected cells, i.e., exhibiting a nuclear and cytoplasmic cellular distribution (Fig. 2A). Fifteen other cell lines were found to express ORF4p, but its localization was perinuclear and probably in the Golgi apparatus. The 3A14 cell line and four of the fifteen other cell lines (all named Vero-IE4) were selected for further characterization. The expression of the ORF4p was examined by Western blot analysis and detected as a 52 kDa band as in VZV-infected cells (Fig. 2B). Finally, transfection assays were undertaken to verify functional activity of the ORF4p expressed in the stable cell lines, using the pTKCAT reporter plasmid containing the VZV thymidine kinase (TK) gene promoter. Figure 2C shows that the CAT activity directed by the TK promoter was increased 5- to 10-fold in Vero-IE4 cell lines. All these results show that the cell lines constructed express a functionally active ORF4p.

The ORF62p was detected by indirect immunofluorescence exclusively in the nucleus of VZV-infected and transiently transfected cells. It was also found in the nucleus of 1% of ORF62-transformed cell lines when immunostaining using the avidin-biotin amplification system as described in the Methods section was carried out (Fig. 3A). Western immunoblot analysis undertaken with the same antibody allowed the detection of a 175 kDa protein in ORF62p-expressing cell lines (named Vero-IE62) (Fig. 3B). The functionality of the ORF62p expressed in these cell lines was examined by transfection assays with the p61CAT target plasmid. In ORF62p-expressing cell lines, CAT activity driven by ORF61 promoter was increased 2- to 3-fold while a 10-fold transactivation VZV ORF61 promoter by ORF62p was previously shown in transiently transfected Vero cells (Fig. 3C). This lower transactivating efficiency recorded in the ORF62p-expressing cell line compared to transient transfection assay can be easily explained by the fact that ORF62p acts as a transactivator at low concentrations, while transactivation decreased at higher concentrations as previously described [Baudoux et al., 1995].

The infection of stable ORF4p- or ORF62p-expressing cell lines with the OKA strain of VZV was carried out as described for the transient expression experiments by using cell-free virus (1.7×10^3 PFU/dish), and subcultures were realized by mixing infected and uninfected cells at a ratio of 1:4 to 1:15, depending upon the CPE level. Infected cultures were passaged 10 times and comparative production of VZV gE antigen was monitored at each passage (Fig. 4A).

Fig. 2. (Figure on previous page). Characterization of ORF4p-expressing cell lines. **A:** Immunofluorescence analysis of ORF4p expression in the 3A14 cell line. Detection of the ORF4 gene product was performed using a polyclonal rabbit antiserum directed against the ORF4 protein. Immune complexes were detected with an FITC-conjugated swine antiserum against rabbit immunoglobulins. Magnification ×500. **B:** Western blot analysis of ORF4p expression in various Vero-IE4 cell lines using a polyclonal rabbit antiserum directed against the ORF4 protein. Each lane contains lysate from 1.2×10^6 cells. A 52 kDa band is seen in VZV-infected Vero cells and in ORF4p-expressing cell lines (Vero-IE4). **Lane 1,** 3A14; **lane 2,** 4D14; **lane 3,** 4D44; **lane 4,** 5C14; and **lane 5,** 4AS cell lines. **C:** Transactivating activity analysis of ORF4p-expressing cell lines on VZV TK target promoter. ORF4p-expressing cells were transfected with 2 μg of pTKCAT by lipofection. Cells were harvested 48 hr post transfection and CAT activities were determined by diffusion assay as described by Neumann et al. [1987]. CAT activity is expressed in fold induction relative to the uninduced value, obtained with the reporter plasmid alone in the absence of an effector plasmid, arbitrarily set to 1.0. Lane "pRcORF4+pTKCAT" shows transactivation of TK promoter by ORF4p in transiently transfected Vero cells; **lane 1,** 3A14; **lane 2,** 4D14; **lane 3,** 4D44; **lane 4,** 5C14; and **lane 5,** 4AS cell lines.



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During the first viral passages, an increase in viral antigen level was observed, reaching one to three times that in control cells with a CPE increase in transformed cell lines. Progressively, the CPE level rose to 80–90% of the cell culture after only 1 to 2 days in the transformed cell lines, while 2 to 3 days were necessary to reach 80% CPE in control Vero cells. From passages five to eight after OKA inoculation, the increase in the gE antigen level generally reached a maximum of five to 12 times and two to 10 times that in control cells in ORF4p-expressing and ORF62p-expressing cell lines, respectively; suggesting overproduction of virion components in transformed cells in comparison to control infection in Vero cells (Fig. 4A). These experiments were also carried out with the VZV wild type strain. gE antigen levels were similar to those measured for the OKA strain infections and reached up to 15 times those in control cells (data not shown). These results show that VZV replication was indeed boosted in both ORF4p- and ORF62p-expressing Vero cell lines. Meanwhile, the enhancement of the gE antigen level varied from three to 49 times and from three to 31 times for the ORF4p- or ORF62p-expressing cell lines, respectively.

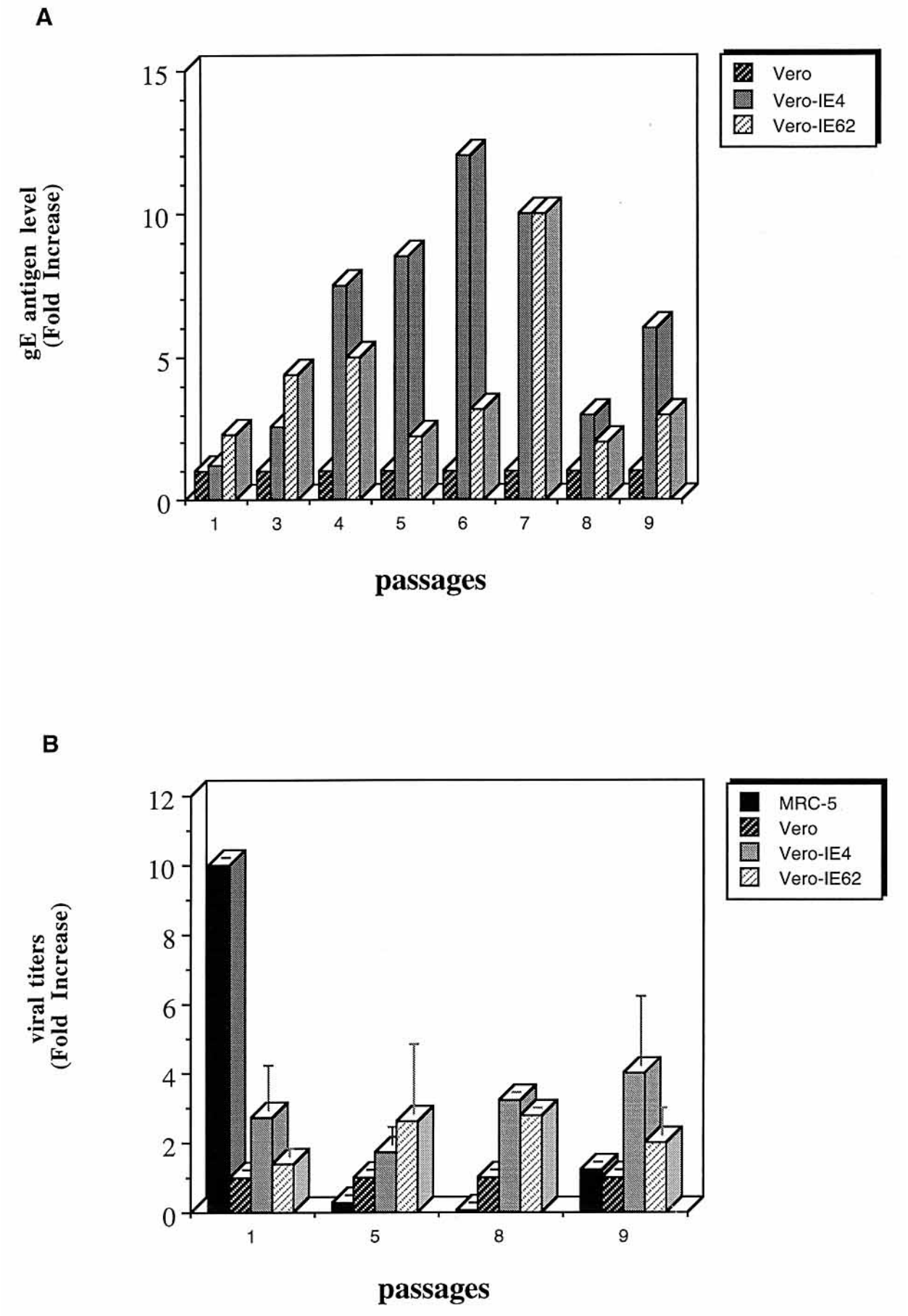
Varicella-Zoster virus has a narrow host range, infecting essentially primate and human cell lines such as the MRC-5 cell line. In order to demonstrate the potential interest of stably transformed Vero cell lines expressing viral proteins, including continuous cell line characteristics, we compared the infection of MRC-5 and Vero-infected cell cultures, using the same procedure as described above. It was observed that gE antigen levels, during the first passages, were higher in MRC-5 infected cells. This was probably due to the adaptation to Vero cells of a VZV OKA strain grown on MRC5 cells. Progressively, gE antigen levels became higher in ORF4p- and ORF62p-expressing cells, suggesting that VZV OKA was overproduced in these cells (data not shown).

Virus titration was also undertaken in order to show the correlation between an increase in gE level and viral production. Figure 4B shows the stimulation of the viral titers in Vero, ORF4p- or ORF62p-expressing Vero cells and in MRC-5 cells. As for gE antigen quantification, it was observed that viral titers, during the first passages, were higher in MRC-5 infected cells and that, progressively, they became higher in ORF4p- and ORF62p-expressing cells. Indeed, at passage 9, in ORF4p-expressing cells, viral titers were five and 12.5 times higher than those obtained for Vero and MRC-5 cells respectively. For ORF62p-expressing cells, viral titers were 1.4 and 3.5 times higher than those obtained for Vero and MRC-5 cells respectively, demonstrating that the enhanced gE production detected above correlated with an increased viral titer.

Fig. 3. (Figure on previous page). Characterization of ORF62p-expressing cell lines. **A:** Immunochemical analysis of ORF62p expression in the 10C562 cell line. Detection of ORF62 gene product was performed using a polyclonal rabbit antiserum against the ORF62p. Using Vero cells as controls of the staining **(picture a)**, immune complexes were detected in ORF62p-expressing samples **(picture b)** with a biotinylated swine antiserum against rabbit immunoglobulins, followed by the ABC-AP amplification system. ORF62p-expressing cells are specified by arrowhead. Magnification ×500. **B:** Western blot analysis of ORF62p expression using a polyclonal rabbit antiserum directed against the ORF62p. Each lane contains lysate from 1.2×10^6 cells. A 175 kDa protein appears in transformed cell lines (Vero-IE62) as well as in Vero cells transiently transfected with pRcORF62 plasmid. **Lane 1,** 10A562; **lane 2,** 10C262; **lane 3,** 10C562, and **lane 4,** 10C662 cell lines. **C:** Transactivating activity analysis of ORF62p-expressing cell lines on VZV ORF61 target promoter. ORF62p-expressing cells were transfected with 2 μg of p61CAT by lipofection. Cells were harvested 48 hr post transfection and CAT activities determined by diffusion assay as described by Neumann et al. [1987]. CAT activity is expressed in fold induction relative to the uninduced value, obtained with the reporter plasmid alone in the absence of an effector plasmid, arbitrarily set to 1.0. Lane "pRcORF62+p61CAT" shows transactivation of ORF61 promoter by ORF62 protein in transiently transfected Vero cells, **lane 1,** 10A562; **lane 2,** 10C262; **lane 3,** 10C562; and **lane 4,** 10C662 cell lines.

DISCUSSION

The role of two strong VZV transactivating proteins, the ORF4p and ORF62p, was studied in the course of viral infection. Cell lines expressing functionally active ORF4p and ORF62p enhance viral production and subsequent viral spread in the culture. These observations were deduced from gE antigen quantification and confirmed by virus titration. ORF62p- and ORF4p-expressing cell lines produce 1.4 to 5 times more viral particles than do control Vero cells, but we were not able to obtain a titer exceeding 10^3 PFU/ml. Indeed, it is well known that VZV spreads by direct cell to cell contact and that no infectious VZV particles are released in the cell culture supernatant. Therefore, cell-free virus is difficult to obtain. This difficulty led us to find an alternative way to quantify the level of infection in infected cultures. Kinetics of gE expression suggest that there is, for each cell line and for each passage of the infected cultures, an optimal time to harvest the cells when gE levels and virus titers are at their maximum (data not shown). This observation might explain variations in the increase in gE antigen and VZV titers observed with our standardized procedure. It must be noted that the final virus yield might also have been increased by the higher dilution factor used each time transformed cell lines were subcultured. Furthermore, in most experiments, stimulation of viral production was higher in ORF4p-expressing compared to the ORF62p-expressing cells. The results suggest that the differences in infection level of these two cell lines appear to reflect the percentage of expressing cells or the level of expressed protein, themselves perhaps influenced by copy number and arrangement of integrated plasmid sequences in the transformed cell lines. Furthermore, it may be noted that three passages of the OKA virus were necessary to observe an increase in gE levels in ORF4p- or ORF62p-expressing cell lines, while the increase in gE levels was immediately obvious when infected cells were transiently transfected. This phenomenon might be due to the fact that 10% of the transiently transfected cells expressed the VZV protein, while only 1 to 3% of expressing cells were detected among transformed cells. The same situation was observed for an HSV-1 IE protein in stable transfection asays. A recent immunofluorescence



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study showed that the percentage of cells expressing detectable HSV ICP27 (a homolog of VZV ORF4p) was quite low (<0.5% of the total). However this low expression was overcome by infecting cells with an HSV-1 ICP27 deletion mutant which enhances the transient expression of IE promoter [Mears et al., 1995]. To explain the mechanisms responsible for this increased infection level, we suggest that more ORF4p or ORF62p, produced during viral transcription and cell transcription from integrated ORF4- or ORF62-expressing plasmids, would be packaged by virions during viral assembly and may play a key role in efficiently initiating the next cycle of infection. In the same context, it has been postulated that the abundance of ORF62p in the virion may be related, in part, to a critical function of the protein during the IE stage of infection [Kinchington et al., 1992]. Another plausible explanation is that the first steps of viral replication might take place earlier and could be more efficiently initiated due to the presence of an increased cellular amount of constitutive viral proteins, such as ORF4p and ORF62p. This could be verified by analyzing the immediate-early gene kinetics in infected ORF4p- or ORF62p-expressing cell lines in comparison with infected control Vero cells.

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Fig. 4. (Figure on previous page). Analysis of VZV OKA infection in ORF4p- or ORF62p-expressing cell lines. **A:** Comparative production of gE antigen. 1.3×10^6 Vero, Vero-IE4 or Vero-IE62 cells were infected by inoculation of 1.7×10^3 PFU of VZV OKA strain and infected cultures passaged 10 times. Total infected cell extracts were monitored for gE antigen production by dot-blotting using anti-gE monoclonal antibody VL-8 as described in the Methods section. Data are presented as fold increase in the amount of gE antigen relative to control value, obtained with infected Vero cells. These experiments were repeated five times and a representative experiment is shown. **B:** VZV OKA titration. For passages 5, 8, and 9, three infected cultures of each cell line were followed. Two of these were used to determine viral titers and one to quantify VZV gE antigen. Cell-free virus was prepared as described in the Methods section. Serial dilutions of the virus suspension were inoculated to MRC-5 monolayers. Titers were determined after immunostaining of the MRC-5 cells using anti-gE monoclonal antibody VL-8 and are expressed as the increase in viral titers obtained from control infected Vero cells. Mean values and standard errors are presented.

REFERENCES

Baudoux L, Defechereux P, Schoonbroodt S, Merville M-P, Rentier B, Piette J (1995): Mutational analysis of Varicella-Zoster virus major immediate-early protein IE62. Nucleic Acids Research 23:1341–1349.

Cabirac GF, Mahalingam R, Wellish M, Gilden DH (1990): Transactivation of viral thymidine kinase promoters by proteins encoded by Varicella-Zoster virus open reading frame 61 and 62. Virus Research 15:57–68.

Cohen JI, Heffel D, Seidel K (1993): The transcriptional activation domain of Varicella-Zoster virus open reading frame 62 protein is not conserved with its Herpes Simplex virus homolog. Journal of Virology 67:4246–4251.

Davison AJ, Scott JE (1986): The complete DNA sequence of Varicella-Zoster virus. Journal of General Virology 67:1759–1816.

Defechereux P, Melen L, Baudoux L, Merville-Louis M-P, Rentier B, Piette J (1993): Characterization of the regulatory functions of Varicella-Zoster virus open reading frame 4 gene product. Journal of Virology 67:4379–4385.

Disney GH, Everett RD (1990a): A Herpes Simplex virus type 1 recombinant with both copies of the Vmw175 coding sequences replaced by the homologous Varicella-Zoster virus open reading frame. Journal of General Virology 71:2681–2689.

Disney GH, McKee TA, Preston CM, Everett RD (1990b): The product of Varicella-Zoster virus gene 62 autoregulates its own promoter. Journal of General Virology 71:2999–3003.

Everett RD (1984): Transactivation of transcription by Herpes virus gene products: Requirement for two HSV-1 immediate-early polypeptides. EMBO Journal 3:3135–3141.

Felser JM, Kinchington PR, Inschauspe G, Straus SE, Ostrove JM (1988): Cell lines containing Varicella-Zoster virus open reading frame 62 and expressing the "IE" 175 protein complement ICP4 mutants of Herpes Simplex virus type 1. Journal of Virology 62:2076–2082.

Inschauspe G, Ostrove JM (1989): Differential regulation by Varicella-Zoster virus (VZV) and Herpes Simplex virus-1 transactivator genes. Virology 173:710–714.

Inschauspe G, Nagpal S, Ostrove JM (1989): Mapping of two Varicella-Zoster virus-encoded genes that activate the expression of viral early and late genes. Virology 173:700–709.

Kinchington PR, Hougland JR, Arvin AM, Ruyechan WT, Hay J (1992): The Varicella-Zoster virus immediate-early protein IE62 is a major component of the virus particle. Journal of Virology 66: 359–366.

Ling P, Kinchington PR, Sadeghi-Zadeh M, Ruyechan WT, Hay J (1992): Transcription from Varicella-Zoster virus gene 67 (glycoprotein IV). Journal of Virology 66:3690–3698.

Moriuchi H, Moriuchi M, Smith HA, Straus SE, Cohen JI (1992): Varicella-Zoster virus open reading frame 61 protein is functionally homologous to Herpes Simplex virus type 1 ICP0. Journal of Virology 66:7303–7308.

Moriuchi M, Moriuchi H, Straus SE, Cohen JI (1994): Varicella-Zoster virus (VZV) virion associated transactivator open reading frame 62 protein enhances the infectivity of VZV DNA. Virology 200: 297–300.

Moriuchi H, Moriuchi M, Smith HA, Cohen JI (1994): Varicella-Zoster virus open reading frame 4 protein is functionally distinct from and does not complement its Herpes Simplex virus type 1 homolog, ICP27. Journal of Virology 68:1987–1992.

Mc Kee TA, Disney GH, Everett RD, Preston CM (1990): Control of expression of the Varicella-Zoster virus major immediate-early gene. Journal of General Virology 71:897–906.

Nagpal S, Ostrove JM (1991): Characterization of a potent Varicella-Zoster virus encoded trans-repressor. Journal of Virology 65: 5289–5296.

Neumann JR, Morency CA, Russian KO (1987): A novel rapid assay for chloramphenicol acetyltransferase gene expression. Biotechniques 5:444.

Perera LP, Mosca JD, Ruyechan WT, Hay J (1992): Regulation of Varicella-Zoster virus gene expression in human T lymphocytes. Journal of Virology 66:5298–5304.

Perera LP, Mosca JD, Sadeghi-Zadeh M, Ruyechan WT, Hay J (1992): The Varicella-Zoster virus immediate early protein, IE62, can positively regulate its cognate promoter. Virology 191:346–354.

Perera LP, Mosca JD, Ruyechan WT, Hayward GS, Straus SE, Hay J (1993): A major transactivator of Varicella-Zoster virus, the immedi-

ate-early protein IE62, contains a potent N-terminal activation domain. Journal of Virology 67:4474–4483.

Sacks WR, Greene CC, Aschman DP, Schaffer PA (1985): Herpes simplex virus type 1 ICP27 is an essential regulatory protein. Journal of Virology 55:796–805.

Snoeck R, Schols D, Sadzot-Delvaux C, Cloes JM, Andrei G, de Clercq E, Piette J, Rentier B (1992): Flow cytometric method for the detection of gE antigen of Varicella-Zoster virus and evaluation of anti-VZV agents. Journal of Virological Methods 38:243–254.